Characterizing combination autophagy inhibition and exogenous stress in murine triple negative breast cancer

Jack Lawton

Honors Thesis
Department of Nutrition
Gillings School of Global Public Health
University of North Carolina at Chapel Hill

April 27, 2018

Approved by:

_____
Dr. Stephen D. Hursting (Advisor)

_____
Dr. Ciara O' Flanagan (Second Reader)

Abstract

Background: Triple negative breast cancer represents a significant treatment challenge due to its inherent aggressiveness and lack of targetable receptors, consequently generating a need for novel treatment approaches. Autophagy, a self-degradative pathway that provides metabolic substrates and recycles damaged proteins and organelles, has been implicated in supporting cancer survival, progression towards metastasis, and chemotherapeutic resistance. Autophagy inhibition has exhibited weak effects on cancer by itself but provides a quality therapeutic target in combination with agents that exploit autophagy deficient cell vulnerabilities, classically through nutrient or chemotherapeutic stress. However, the success of autophagy inhibition in combination therapies has been shown to be highly tumor type, stage, and oncogene dependent, thus making researching the effect of autophagy and exogenous stress on Triple negative breast cancer an important avenue for continuing autophagy and cancer research.

Methods: In order to characterize autophagy deficient metastatic TNBC in vitro, ATG5, an essential autophagy protein, was knocked out using a CRISPR/Cas 9 construct in both a mesenchymal mouse derived TNBC cell dubbed the M-Wnt and in a metastatic line developed from M-Wnt lung metastases, called the metM-Wnt^{lung}. Experiments using both Wild-Type and autophagy deficient lines assayed relative growth, energy generation potential, EMT phenotype, and response to stress in the form of chemotherapeutics and nutrient starvation.

Results: Relative to their autophagy competent counterparts, Atg5-/- cells showed decreased growth and significantly altered metabolism, displaying high basal energetics but reduced maximum energy production. Autophagy inhibition alone also resulted in downregulation of EMT regulators Twist, Snail, and Slug, and morphologically exhibited signs of an EMT reversion. Growth and metabolic effects were not observed across all exogenous stressors; only serum starvation and doxorubicin treatment appeared to act synergistically with autophagy inhibition to reduce cancer growth. Reactive oxygen species induced by doxorubicin and an inability to balance redox stress via an antioxidant response (NRF2) may underlie synergistic chemotherapy effects.

Conclusions: These findings suggest that autophagy inhibition induces broad cellular changes, including metabolic alterations that strengthen specific nutrient and chemotherapeutic treatments. Further investigation of this model in vivo and identification of a molecular target to maximize autophagy inhibition effects have potential to lead to practical, effective adjuvant or neoadjuvant combination therapies.

Table of Contents

Acknowledgements

I'd like to thank Dr. Stephen Hursting and the entire Hursting lab for providing me with an incredible opportunity to learn in a challenging and supportive environment. I would also like to thank Dr. Ciara O'Flanagan for showing me what it truly means to be a scientist, and without whom I wouldn't have had the foundation and guidance to perform this independent study. Lastly, I'd like to thank my family for their unwavering faith in my goals and for always being proud of me, no matter how small the achievement.

Introduction

Breast Cancer Prevalence and Mortality

Breast cancer is the most prevalent cancer and second leading cancer-related cause of death in women, amassing an estimated 252,710 new diagnoses and 40,610 deaths in 2017[1]. It makes up to 30% of all female cancer diagnoses, and while 5 year survival rates for Stage 0 or stage 1 breast cancers are nearing 100%, treatment of advanced breast cancer has not been as successful[1,2]. Widespread mammography screening and the advent of molecular diagnostics have improved overall breast cancer death rates at a mark of 1.9% every year from 2004 to 2013, but progress on certain molecular subtypes has been slow[2]. Breast cancers can be broken down into multiple subtypes by DNA and gene expression, with the major subtypes being luminal A, luminal B, HER-2 enriched, and Triple Negative[3]. Each subtype confers a different prognosis and different treatment options, and Triple negative breast cancer (TNBC) represents a significant challenge in current breast cancer treatment.

Triple negative breast cancer

Triple negative breast cancer makes up 12% of total breast cancer cases and is classified by the lack of estrogen receptors (ER-), progesterone receptors (PR-), and HER2/neu expression (HER2-)[4]. Accordingly, TNBC also lacks the highly effective hormonal and monoclonal antibody therapies that have been used to target breast cancers expressing these receptors. Compared to the other subtypes of breast cancer, patients with Triple negative breast cancer were more likely to receive chemotherapy, have a shorter time to recurrence, display more aggressive tumors, and exhibit worse overall survival[5–7]. Triple negative breast cancer also embodies a public health concern in the form of a racial health disparity, as black women are twice as likely white women to be diagnosed with TNBC and had twice the 7 year risk of death from Stage 1 TNBC as white women[4,8,9]. Surgery and chemotherapy have been the traditional treatment

approaches for TNBC, but as oncology and molecular biology have advanced, the field is looking to replace these highly damaging strategies with targeted, low toxicity treatments.

Genetically, Triple negative breast cancer is more problematic than other breast cancers. TNBC is highly heterogeneous, and unlike the other subtypes with common oncogene drivers, the only genes mutated in 10% or more of TNBCs are TP53 and PIK3CA[10]. Thus, there are few actionable pathways that can be targeted across all TNBCs, although combination therapy targeting PI3K/mTOR and RAS/RAF/MEK has shown promise in current clinical trials[5,7]. TNBC is also the most likely subset of breast cancer to be claudin-low, a recently defined molecular subtype that identifies cancers as showing low expression of the claudin genes involved in epithelial junctions and is associated with poorer distant metastasis-free survival at 5 years[3,11]. Most importantly, claudin-low breast cancers are known to be enriched in tumor initiating cells and show stem cell phenotypes, the subpopulations implicated in tumorigenesis, tumor progression, and chemotherapeutic resistance[3,12,13]. Future TNBC therapies should have a role in reducing metastasis of these highly aggressive tumors.

Additionally, traditional research on breast cancer and triple negative breast cancer has centered on RAS driven cancers, and the Wnt pathway represents a missed opportunity. Wnt signaling in cancer is well described regulator of a wide variety of processes including differentiation, proliferation, and cell motility and invasion, but is generally understood to drive highly proliferative, aggressive and phenotypes across all cancers when dysregulated[14]. Aberrant Wnt signaling is common in TNBC and associated with poor clinical outcomes within TNBC; patients with abnormal Wnt/β-catenin signaling are more likely to develop lung and brain metastases than with other genetic drivers[11,14].

Thus, there is a strong need for new approaches and novel therapies to treat Triple negative breast cancer in general, and especially a demand to explore avenues for treating highly metastatic and tumor cell enriched Wnt-driven and claudin-low cancers.

Autophagy and Cancer

The relatively recent discovery of autophagy provides a novel, targetable approach for TNBC treatment. Autophagy can be generally classified into three groups: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA)[15,16]. Both microautophagy and CMA have important cellular roles, but macroautophagy (hereafter referred to as autophagy) is the major catabolic pathway implicated in multiple pathogeneses, particularly in cancer[17]. In the process of autophagy, cellular contents are sequestered in a double membrane vesicle and then fused to lysosomes for degradation. Its canonical effect is two-fold: autophagy-mediated degradation provides macromolecules under nutrient stress to fuel ATP production and biosynthesis as well as recycling damaged proteins and organelles as a stress response. Autophagy is induced by hypoxia, ER stress, nutrient starvation, especially amino acid deprivation, and provides a way for all cells, and especially cancer cells, to respond to intracellular and extracellular change[18,19]. The full mechanisms surrounding autophagy regulation and its effects on cancer cells are incomplete, but the phosphatidylinositol 3-kinase/mammalian target of rapamycin (PI3K/mTOR) pathway is recognized as the primary regulator of autophagy; mTOR integrates nutrient, growth factor, and stress signals and mTOR activation inhibits autophagy in order to promote growth[20].

Autophagy's role in cancer is highly complex and dependent on cancer type, tumor stage, and oncogene, making research on autophagy in less-well studied cancer phenotypes a necessity[17,20,21]. However, autophagy is generally considered to suppress early stages of cancer

but promote survival and progression towards metastasis in solid tumors and later stage cancer[17,23]. Autophagy activation reduces cellular stress and DNA damage, therefore suppressing tumorigenesis and reducing genomic instability[24,25]. These effects led to initial understandings of autophagy as a tumor suppressor, but it seems that cancer preventative effects are most salient in tumor formation, while the pro-survival effects of autophagy dominate once a solid tumor is established[26,27]. Autophagy promotes survival of established tumors in hypoxic, nutrient-poor, or otherwise stressful environments, and is known to support the survival of dormant tumor cells, disseminating tumor cells in circulation, and stem-like subpopulations of tumor cells responsible for invasion and chemotherapy resistance[24,28,29].

Perhaps most importantly, autophagy has been implicated as an important player in cancer progression towards metastasis, the primary cause of cancer related death[30,31]. Evidence indicates direct regulation of EMT, tumor cell migration, and invasion by autophagy, and in certain "autophagy-addicted" cancers, like KRAS mutated lines, autophagy has been shown to be necessary for metastasis[21,32,33]. Considering that fewer than 30% of women with breast cancer metastases survive 5 years post-diagnosis and nearly all women with metastatic TNBC will die of cancer related complications, targeting autophagy in order to impact metastasis could prove critical to improving breast cancer mortality in the future[5].

Autophagy Inhibition Combination Therapy

As autophagy plays a crucial role in a variety of cellular processes, its inhibition provides multiple metabolic vulnerabilities that can be exploited in cancer therapy. In much of the seminal autophagy research, one of the 30 core autophagy proteins, typically ATG5, ATG7, or ATG12, is genetically knocked out or silenced in mice after-birth, as nearly all $ATG^{-/-}$ genetically engineered mouse models result in neonatal death[34]. Inhibition is pharmacologically achieved

with hydroxychloroquine or 3-methyladenine most commonly, but their inhibition is weak compared to genetic inhibition[35,36]. However, hopes of potent novel inhibitors have sustained the push towards using autophagy inhibition in combination to sensitize cancer before or during chemotherapy treatment. Specifically, combining autophagy inhibition with nutrient and chemotherapeutic stressors hopes to take advantage of the lack of autophagy's two major functions: provision of metabolic fuel under stress and responding to cell stress by clearing intracellular damage.

Altering nutrient intake was indicated as a novel therapy for a host of diseases before cancer was considered, but combining nutrient intake modulation, especially calorie restriction, and autophagy inhibition is yielding positive results in pre-clinical studies. Calorie restriction (CR) has been well demonstrated to be cancer preventative, but recent research has turned towards using CR to sensitize cancer to radiation and chemotherapies[37–39]. *In vitro* work has shown sensitization of autophagy deficient colon cancer cells to glucose restriction[40,41]. More significantly, in a *in vivo* study by Lashinger et al., a combination of calorie restriction and autophagy inhibition resulted in the lowest tumor burden among all given populations[26]. However, there is still gap in knowledge to be filled, as none of the studies were performed on Triple negative breast cancer, and questions remain as to whether autophagy deficient cancers are vulnerable to CR mimetics such as Metformin or IGF1R inhibitors

Autophagy's role in chemotherapeutic resistance is unfortunately as context dependent as its role in cancer, but multiple clinical trials are underway to determine the contexts in which combination chemotherapy and autophagy inhibition are successful[22,35]. Autophagy has been implicated as a mechanism for chemotherapy resistance, as cells can upregulate autophagy to balance redox stress and suppress intracellular DNA and organelle damage[42]. Additionally,

chemotherapy and radiation therapy both are known to induce autophagy upregulation, but this doesn't necessarily indicate a dependence on autophagy[42]. On the other hand, excessive autophagy induced by chemotherapeutics has been associated with cell death and autophagy mediated cell death may be a mechanism through which some chemotherapeutic agents act[22,43].Thus, dependent on the contexts, autophagy inhibition and chemotherapeutic combination therapy can increase killing or protect cells [22,42]. There is no clear answer as to the success of combination chemotherapy autophagy inhibition, but the 12 phase I and II clinical trials and bounds of preclinical studies should illuminate its potential. However, none of the clinical trials and few preclinical studies assess combination therapy effectiveness in TNBC, much less in highly aggressive Wnt driven, claudin-low TNBC.

Goal and Hypotheses

The primary goal of this study was to better understand the effects of autophagy inhibition with and without exogenous stressors in Wnt driven claudin-low Triple negative breast cancer. The study was accomplished through three specific aims, the first aim being to determine the effects of autophagy inhibition alone in the M-Wnt and metM-Wnt^{lung}, specifically in the areas of growth, metabolic ability, and invasive phenotype. Our corresponding hypothesis is that autophagy inhibition alone in high nutrient contexts will cause moderate decreases in growth and oxidative phosphorylation, but few other effects. The second specific aim is to assay cellular changes of autophagy deficient cells in response to exogenous stress in the form of nutrient modulation or chemotherapeutics. As found in a previous *in vivo* study within the lab, we expect a combination of autophagy inhibition and *in vitro* calorie restriction mimic to result in the lowest amount of growth relative to autophagy competent and high nutrient conditions[26]. Additionally, we hypothesize that autophagy inhibition will sensitize cancer to all types of nutrient stress but will not sensitize cancer to all chemotherapeutics, as prior work has shown autophagy inhibition effectiveness with chemotherapeutics to be highly context dependent[21,42,44]. The third specific aim is to elucidate potential mechanisms underlying interactions between autophagy inhibition and exogenous stress. Our hypothesis is that the accumulation of damaged organelles, especially mitochondria, and inability to provide endogenous metabolic fuel place autophagy deficient cancer cells in a state of reduced metabolic plasticity[18]. Adding further stress through restriction of nutrients or the addition of chemotherapeutic agents results in excessive, unbalanced ROS and mitochondrial damage induced apoptosis.

Methods

Generation of Wild-Type and Autophagy Deficient TNBC Cell Lines

Previously described fully in Dunlap et al. 2012[13] and O'Flanagan et al. 2017, the M-Wnt cell line was developed from spontaneous mammary tumors in MMTV-Wnt-1 transgenic mice[45]. The M-Wnt line has mesenchymal morphology, is strongly mammosphere forming, and shows high migratory and invasive ability[13]. The metM-Wnt^{lung} cell line was derived through harvesting of lung metastases in severe combined immunodeficient mice after serial transplantation of M-Wnt cells in 5 generations of mice. metM-Wnt^{lung} cells were analyzed and found to display multiple markers and behaviors of metastatic and TIC enriched populations[45].

Autophagy deficient cell lines of both the M-Wnt and metM-Wnt^{lung} lines were generated via a CRISPR/Cas9 lentiviral vector (Sigma-Aldrich) containing both Puromycin resistance and GFP components. This lentivirus was used to transduce and knockout ATG5, a protein required for autophagy, in both the M-Wnt and metM-Wnt^{lung} cell lines[44]. Post-transduction, cells were selected by culturing in media with Puromycin, and clonal populations were generated from single cells. Clones with strong knockout were screened by GFP expression and ultimately selected by Western Blot (Figure 1A). The populations chosen as the M-Wnt and metM-Wnt^{lung} ATG5$^{-/-}$ lines expressed little to no ATG5 protein and high LC3B-1:LC3B-II ratio, a marker of autophagy induction without formation of autophagosomes[16].

Growth Assays

Long Term Survival

Colony formation assays were used to measure long term survival and ability to form colonies. Cells were seeded into 6 well plates at 1×10^3 cells/well. Plates were incubated and

grown for 14 days with complete, 1% FBS, 1mM, or FA- media. At day 7 for all experiments, media was replaced with fresh media of the respective treatment. At day 14, cells were fixed in 1 mL of 100% methanol for 10 minutes. Methanol was aspirated and replaced with 1 mL 0.5% Crystal Violet in 50% methanol for 30 minutes. Plates were then washed with water and stained colonies were counted by hand.

Short Term Survival & Dose Response Curve

MTT survival assays were used to measure short term survival and dose response to chemotherapeutics. Cells were seeded at 5×10^3 cells/well in a 96 well plate, incubated for 24 hours, then media was replaced with treatment media (1% FBS, 1mM Glucose, FA-, +Chemotherapeutic) or complete media. At 48 hours, cell media was replaced with 100 µL of 0.5 mg/mL of MTT reagent in complete media for 1 hour, covered with aluminum foil. MTT media was then aspirated, 100 µL of DMSO was added, and plates were placed on a microplate shaker at 1,000 RPM for 5 minutes. Using the Cytation 3 Cell Imaging Reader (BioTek) to obtain measurements, absorbance at 690 nm subtracted from absorbance at 595 nm was used as relative measure of survival. All relative survival measurements were normalized to a non-treated control population.

Metabolic Flux Analysis

Cellular bioenergetics were collected using the Seahorse XFe96 Analyzer (Agilent Technologies) with a Mito Stress Test Kit. Cells were seeded at 15×10^3 cells/well in a 96 Well Seahorse XF Microplate for 24 hours with complete media, then media was switched to treatment media (1% FBS, 1mM Glucose, FA-). Following 48 hours of treatment, cells were incubated for 1 h at 37 °C in a CO2-free atmosphere, then media was replaced with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose supplemented Seahorse XF Base Media at pH

7.4. OCR and ECAR were measured following administration of oligomycin (1 μM), carbonyl cyanide-4-(trifluormethoxy) phenylhydrazone (FCCP) (1μM), and antimycin A (3μM)/rotenone (3 μM). Results were normalized to μg protein using a BCA protein assay (Thermo Fisher).

EMT Transcription Factor and Biomarker qPCR

MetM-Wnt^{lung} cells were seeded into 6 wells at 500 × 10^3 cells/well for qPCR with complete RPMI media. After 48 hours, cells were harvested with Trizol reagent (Sigma Aldrich) into 1.5-mL microcentrifuge tubes for storage at -80° C, then RNA was isolated and cDNA synthesized. EMT transcription factors and biomarkers were measured by qPCR using TaqMan™ Gene Expression Assays. Data was collected on a ViiA7 (Applied Biosystems). Gene expression data were normalized to β-actin.

Mitochondria and Reactive Oxygen Species Flow Cytometry

For all experiments, cells were seeded at 350 × 10^3 cells/well in a 6 well plate and grown for 24 hours, at which media was replaced with either complete media or a treatment media as described earlier. Samples were then incubated for 48 hours, after which cells washed with PBS and fluorophore solution was added. All sample data contained at least 50,000 ungated events, and was collected using an Accuri C6 Flow Cytometer (BD Biosciences) and analyzed with FlowJo software (BD Biosciences).

Mitochondrial Membrane Polarization

MitoTracker Red CMXRos (Invitrogen) in complete media was added at 100nm for 30 minutes. Cells were washed with PBS, collected via 0.05% trypsin, and then resuspended in PBS. Relative fluorescence intensity at detector FL-2 was used as a measure of mitochondrial polarization. FCCP was added at 100uM for 30 minutes as a positive control[46].

General ROS

General reactive oxygen species were measured via the Cellular Reactive Oxygen Species Detection Assay Kit, Deep Red Fluorescence (Abcam). 1 µL of ROS Deep Red stock solution per mL of complete media was placed on cells for 30 minutes. Media was aspirated, cells were washed with PBS, collected by 0.5 M EDTA, washed again and then resuspended in PBS. Fluorescence intensity at detector FL-4 was used a measure of general ROS species in the cell. Menadione in complete media at 30 µM for 30 minutes was used as a positive control[47].

Quantifying Mitochondrial Superoxide

A solution of 3 µM MitoSOX Red Mitochonrial Superoxide Indicator (Invitrogen) in complete media was added to cells for 10 minutes. Media was aspirated, cells washed with DPBS, collected using 0.5 M EDTA and washed using DPBS with 2% FBS. Cells were fixed in 2% paraformaldehyde for 10 minutes and then resuspended in 0.5 mL of PBS. Relative fluorescence intensity at detector FL-2 was used as a measurement of mitochondrial superoxide production. A solution of Menadione in complete media at 30 µM for 30 minutes was used as a positive control[47].

Western Immunoblot Analysis

Cells were lysed in RIPA buffer. Forty micrograms of protein were loaded into a 4–15% stain-free gel (Biorad). Proteins were resolved and transferred to a PVDF membrane using a Transblot Turbo transfer unit (Biorad). Membranes were blocked with 5% bovine serum albumin for 1 hour before being incubated overnight at 4 °C with one of the following primary antibodies: rabbit ATG5, rabbit LC3B, rabbit NRF2, mouse PCNA, or mouse anti-β-Actin (Santa Cruz Biotechnology), followed by HRP-conjugated secondary antibodies raised against rabbit or mouse IgG (Sigma Aldrich). Proteins were visualized using a Chemi Doc MP system (Biorad).

Statistical Analysis

All data was analyzed using GraphPad Prism 7 software. Differences between ATG5 Knockouts and Wild-Type were analyzed by Student's t-test and ANOVA using Tukey's multiple comparisons test. $P < 0.05$ was considered statistically significant, indicated by * in figures.

Results

Autophagy Inhibition Alone

Growth and Proliferation

CRISPR/Cas9 knockout of ATG5 is partial in the M-Wnt line and complete in metM-Wnt^{lung}, but both populations show autophagy induction without formation of autophagosomes, as assayed by LC3B cleavage (Figure 1A)[16,48]. Cell growth is decreased in ATG5 knockouts and compounds over time (Figure 1B). Clonogenic assays, a proxy for long term growth, show significant decreases in colony formation over 14 days (Figure 1C,1D).

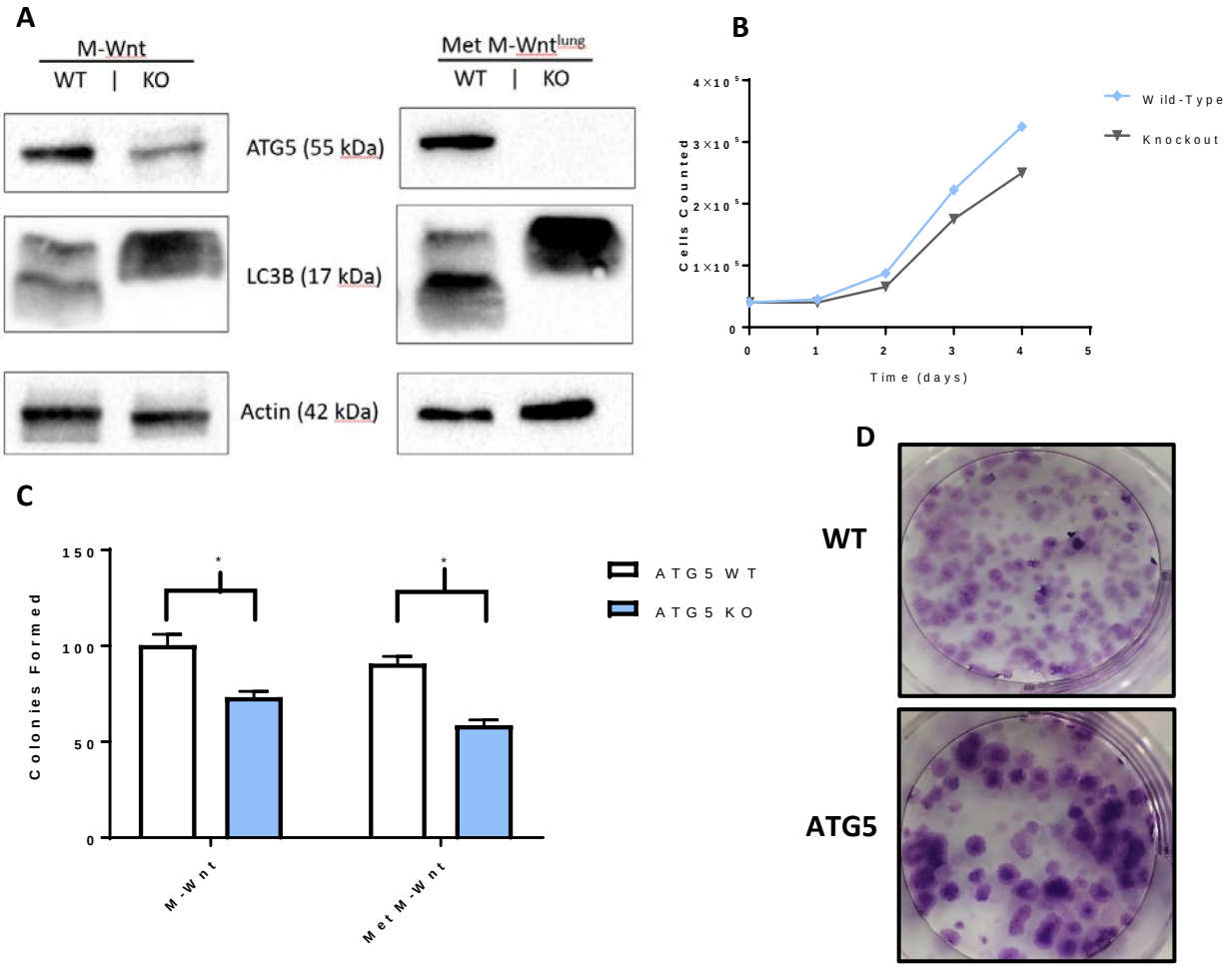
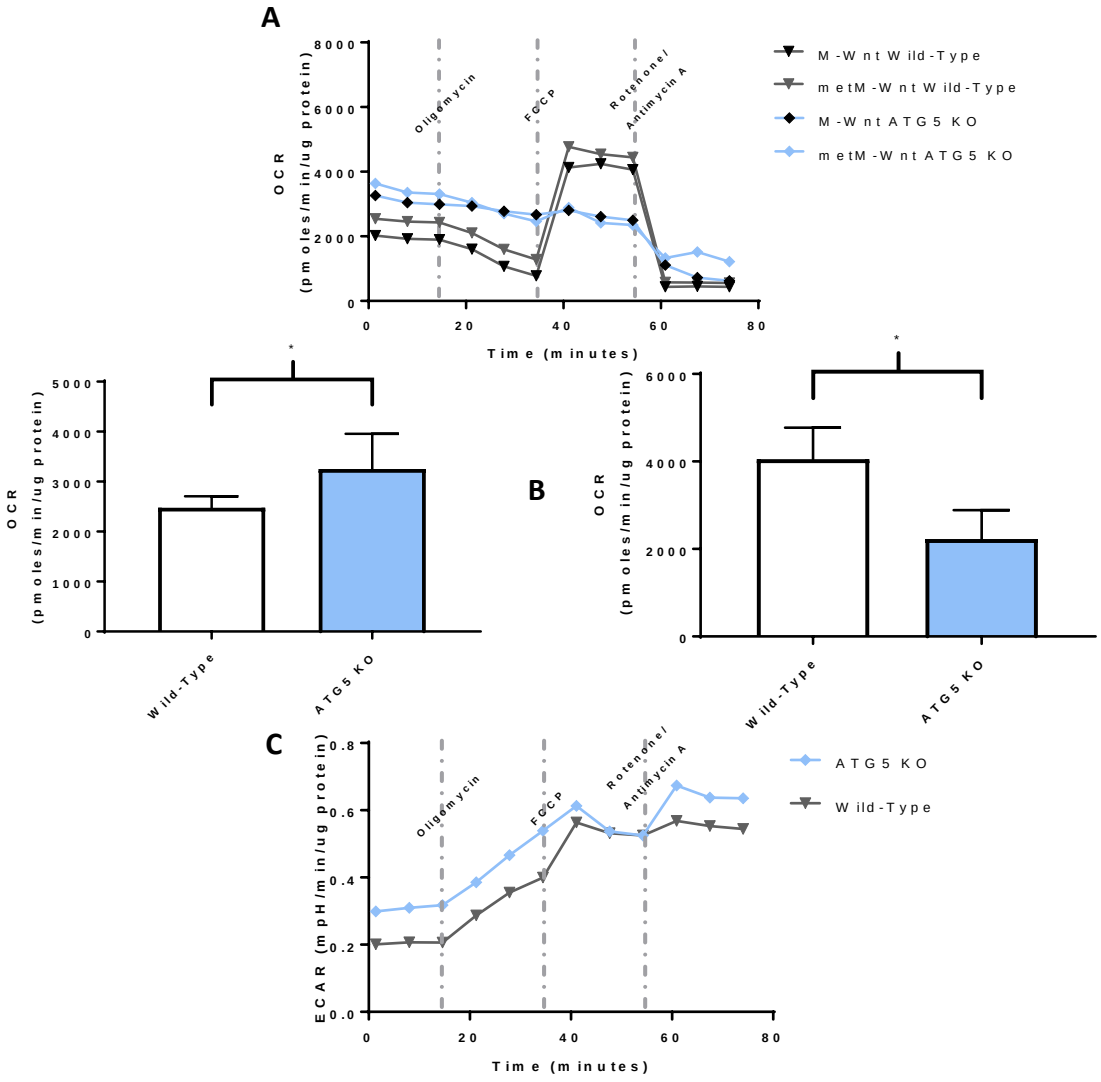


Figure 1. *In vitro* growth effects of autophagy inhibition **(A)** Immunoblot analysis of ATG5 expression and LC3B cleavage **(B)** Growth curve of metM-Wnt^{lung} **(C)** 14-day clonogenic assay **(D)** Respective photos of clonogenic assay

Metabolic Ability

Autophagy deficient cells show significantly increased basal oxygen consumption rate and significantly decreased maximum oxygen consumption (Figures 2A, 2B). Furthermore, ATG5 knockouts do not respond to injections of electron transport chain inhibitors, unlike autophagy competent cells (Figure 2A). Autophagy deficient metM-Wnt^{lung} also display a significantly elevated basal extracellular acidification rate, but similar maximum inducible extracellular acidification rates (Figures 2C, 2D)



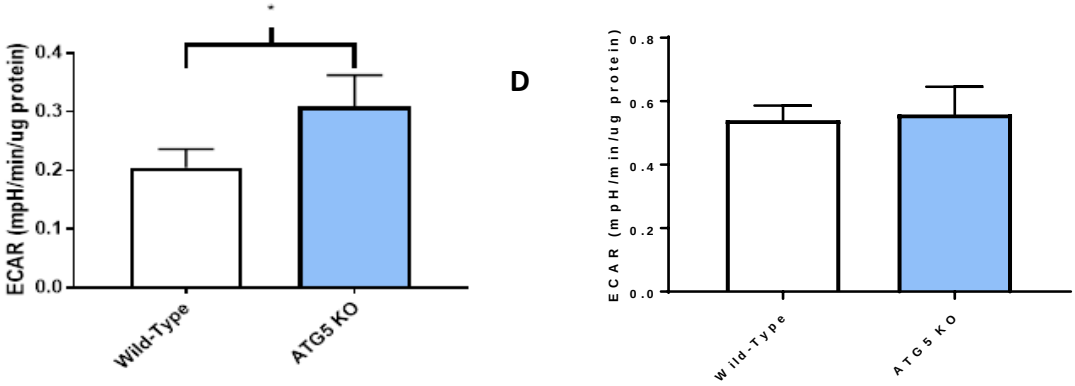


Figure 2. Metabolic effects of autophagy inhibition. **(A)** Oxygen consumption rates in nutrient-replete conditions, FCCP administration establishes maximum respiratory capacity. **(B)** Basal (left) and maximum (right) oxygen consumption rates of metM-Wnt^{lung}. **(C)** Extracellular acidification rate (ECAR) of metM-Wnt^{lung} **(D)** Basal (left) and maximum (right) ECAR of metM-Wnt^{lung}

Morphology and EMT Biomarkers

Wild-Type metM-Wnt^{lung} cells show mesenchymal-like or fibroblast-like morphology with elongated spindle cytoplasm, consistent with human claudin-low breast cancer lines (Figure 3A). Autophagy deficient metM-Wnt^{lung} display epithelial-like morphology, growing with polygonal shapes in sheets (Figure 3B). Real-time PCR yielded significant downregulation of EMT transcriptional factors Snail, Slug, and Twist in autophagy deficient metM-Wnt^{lung} cells relative to Wild-Type cells (Table 1). Additionally, borderline significant changes were found in structural and junction proteins Vimentin, E-Cadherin, and N-Cadherin (Table 1).

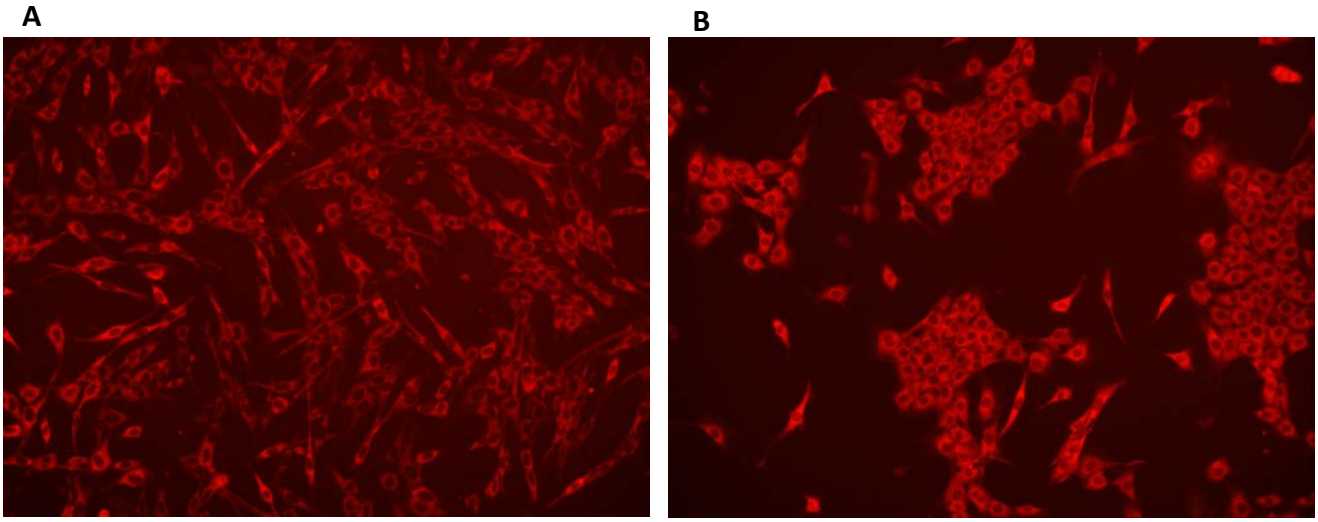


Figure 3. Representative photomicrographs of **(A)** metM-Wnt^{lung} Wild-Type cells and **(B)** autophagy deficient metM-Wnt^{lung} cells

Gene	Fold Change	p-value	Gene Description
Snail (SNAI1)	-1.901	0.0007	Repressor of E-Cadherin. Snail expression in breast cancers is associated with metastasis, tumorrecurrence and poor prognosis[49,50]
Slug (SNAI2)	-1.697	0.0431	E-Cadherin transcriptional repressor
Twist (TWIST1)	-1.418	0.0282	Upregulates N-Cadherin, has a role in promotion of metastasis and CSC maintenance[51,52]
Zeb (ZEB1)	-1.547	0.0712	E-Cadherin transcriptional repressor, promotes malignant progression towards metastasis[53]
Vimentin (VIM)	-1.748	0.0681	Intermediate filament protein expressed ubiquitously in mesenchymal cells, associated with EMT progression[54]
E-Cadherin (CDH1)	1.285	0.1624	Epithelial cell-cell adhesion protein, downregulation is a biomarker for increased invasiveness and metastasis[49]
N-Cadherin (CDH2)	-1.219	0.0598	Protein in neural cell-cell adhesions, a biomarker for mesenchymal phenotype and upregulation associated with endothelial invasion[55]

Table 1. EMT transcription factors and biomarkers altered by ATG5 knockout.

Autophagy Inhibition in Combination with Exogenous Stress:

Growth and Proliferation

48-hour growth in M-Wnt cells is reduced by 1% FBS treatment, however there is no compounding effect on growth with autophagy inhibition (Figure 4A, 4B). In long-term clonogenic assays, 1% FBS in combination with autophagy inhibition works synergistically in suppressing growth, forming only 14% of the colonies that autophagy competent cells in nutrient-replete media do (Figure 4C, 4E). Glucose restriction strongly reduces colony formation over 14 days but has no additive effect with autophagy inhibition (Figure 4D).

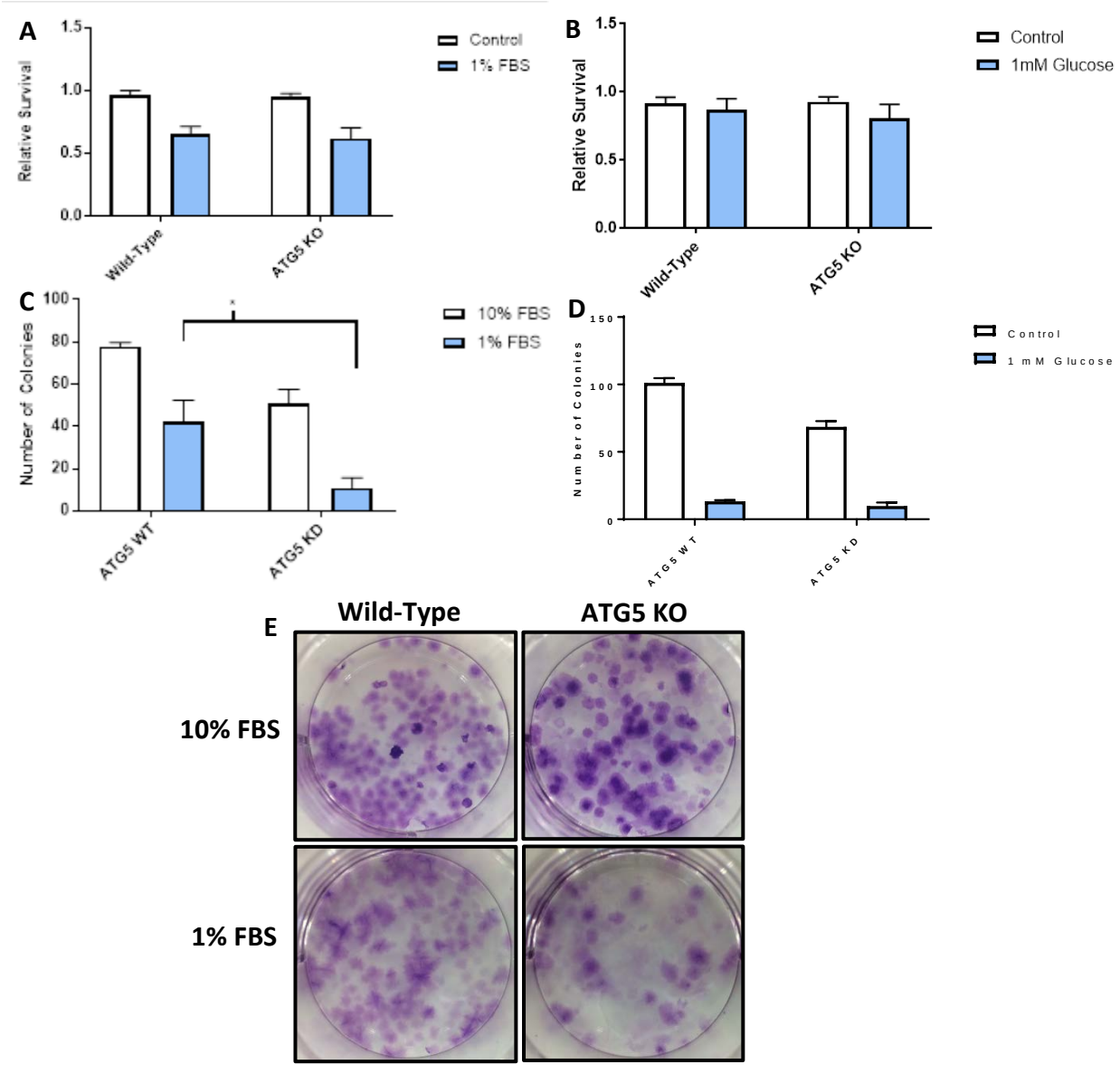


Figure 4. Growth effects of autophagy inhibition in combination with nutrient stress in M-Wnt **(A)** 48 hr MTT survival assay (Control is 10% FBS) **(B)** 48 hr MTT survival assay (Control is 25 mM glucose) **(C)** 14 day clonogenic assay **(D)** 14 day clonogenic assay **(E)** Representative photographs of Figure 4C

Metabolic Ability

ATG5 knockout decreases maximum oxygen consumption rate and extracellular acidification rate across treatments (Figures 5A, 5B). Glucose restriction in autophagy deficient cells significantly decreases maximum OCR but does not display a corresponding rise in ECAR, while 1% FBS does not have a significant effect on either measure (Figures 5A,5B)

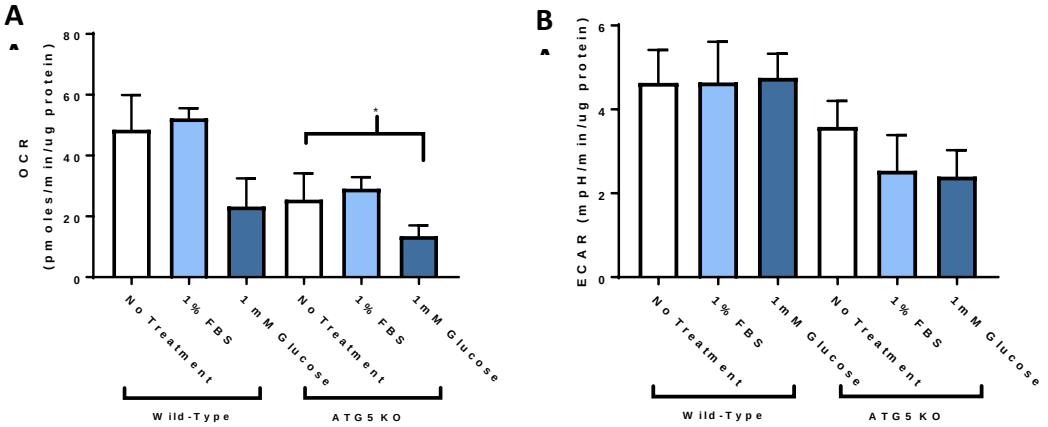


Figure 5. Metabolic effects of combined autophagy inhibition and nutrient stress in metM-Wnt^{lung}. **(A)** Maximum oxygen consumption rate. **(B)** Maximum extracellular acidification rate.

Chemotherapy Response

Autophagy inhibition sensitizes metM-Wnt^{lung} cells to Doxorubicin at multiple concentrations (Figure 6A). No differential survival in MTT assays was found between Wild-Type and ATG5 KO for Paclitaxel, Gemcitabine and calorie restriction mimetics Metformin and BMS-754807, an IGF1R inhibitor (Figure 6B).

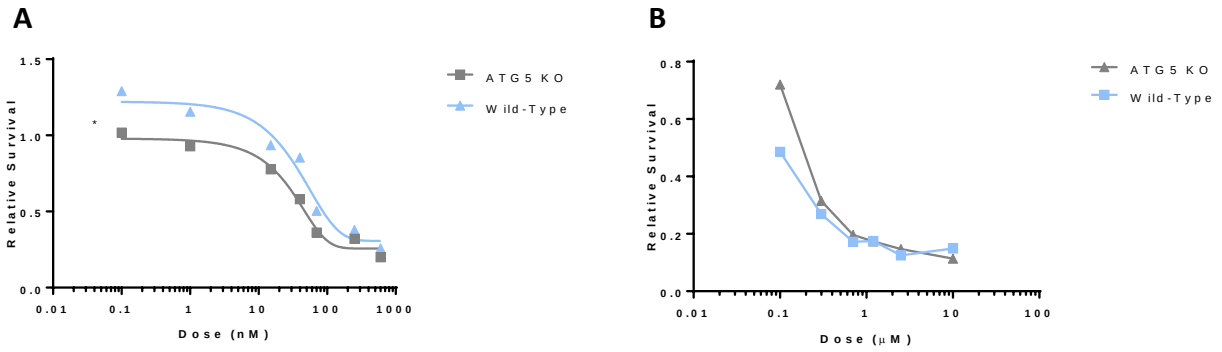


Figure 6. Chemotherapeutic dose response curves in metM-Wnt^{lung}. **(A)** Doxorubicin **(B)** Paclitaxel, representative of non-significant chemotherapeutic agents

Mitochondria and Reactive Oxygen Species Analysis:

Mitochondrial Membrane Polarization

Staining with CMXRos reveals a lower mitochondrial membrane polarization under nutrient-replete conditions in autophagy deficient metM-Wnt^{lung} (Figure 7A). Doxorubicin treatment induces higher mitochondrial membrane polarization in Wild-Type cells, but only marginally in ATG knockouts (Figure 7B).

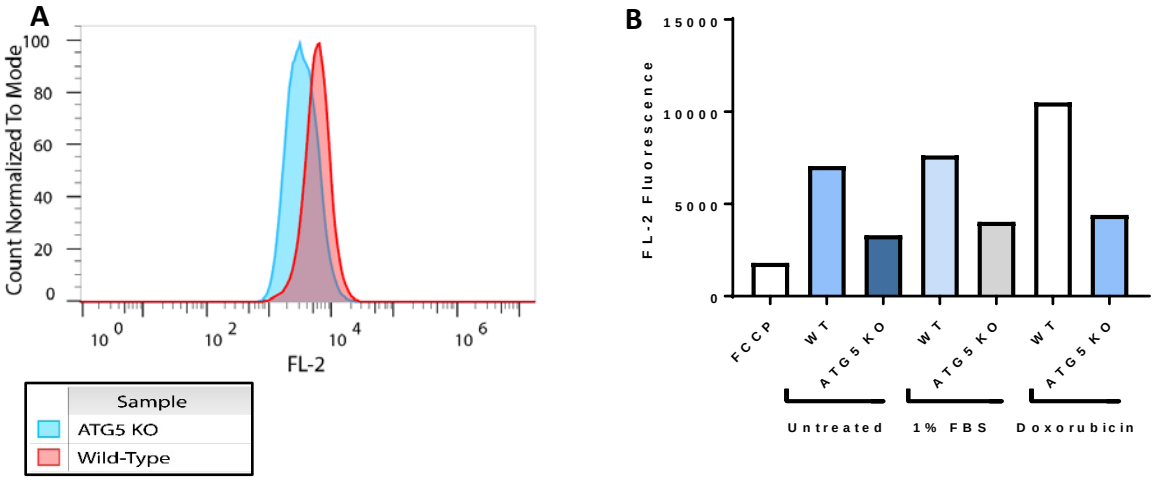


Figure 7. Effects of autophagy inhibition and exogenous stress on mitochondrial membrane polarization. **(A)** Representative frequency histogram of cellular fluorescence; lower fluorescence indicates lower mitochondrial membrane polarization **(B)** Median fluorescence intensity. Doxorubicin treatment at 55 nm.

General and Mitochondrial ROS

Autophagy deficient metM-Wnt^{lung} display lower general ROS than Wild-Type cells under nutrient replete conditions (Figure 8A). In Doxorubicin treatment, but not in Gemcitabine treatment, ATG5 knockouts have higher general reactive oxygen species than Wild-Type cells (Figures 8B, 8C). Autophagy deficient metM-Wnt^{lung} show lower mitochondrial ROS, primarily superoxide, in both nutrient replete and serum starvation conditions (Figure 8D).

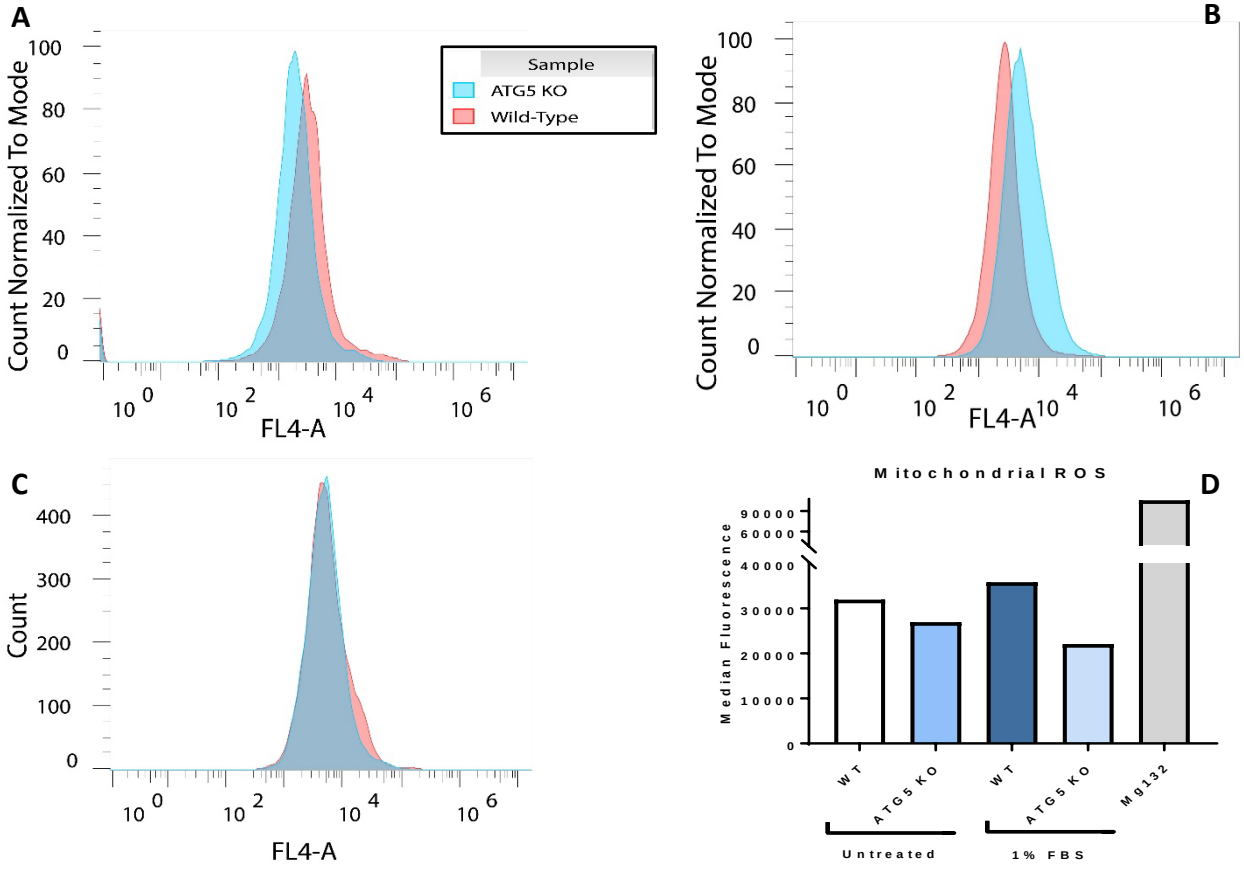


Figure 8. Reactive oxygen species presence in response to chemotherapeutic stress in metM-Wnt^{lung}. **(A)** Frequency histogram of general ROS in nutrient-replete conditions **(B)** General ROS after 48 hr Doxorubicin treatment at 55 nm **(C)** General ROS after 48 hr Gemcitabine treatment at 30 nm **(D)** Mitochondrial superoxide production, median fluorescence intensity reported

Intracellular Stress Response

ATG5 knockout M-Wnt cells display high NRF2 induction basally whereas Wild-Type cells show little NRF2 protein. Additionally, autophagy deficient cells are unable to continue inducing NRF2 under 48 hour and 14 day folic acid deprivation, while Wild-Type cells can maintain NRF2 protein (Figure 9).

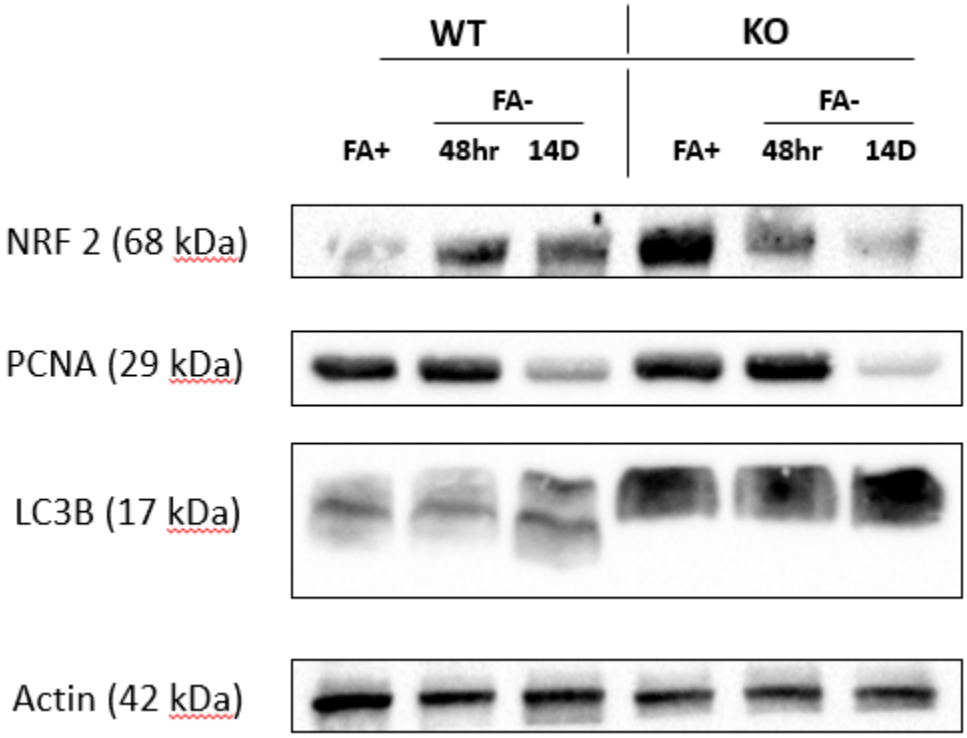


Figure 9. Antioxidant response to folic acid deprivation. Inserted Western Blots indicate protein expression levels of NRF2, PCNA, and LC3B. Data are representative of at least 3 independent experiments.

Discussion

This study aimed to better understand autophagy inhibition's *in vitro* effects on Wnt driven, claudin-low Triple negative breast cancer and to determine if autophagy inhibition in combination with nutrient or chemotherapeutic stress is a viable therapy to be tried *in vivo* murine models. The study produced evidence displaying autophagy inhibition's widespread effects on cellular behavior and identified two specific stressors, serum starvation and Doxorubicin, that work synergistically with autophagy inhibition. Mechanisms behind Doxorubicin's effect appear to be reactive oxygen species related, however a mechanism for serum starvation's effect was not directly identified.

Autophagy deficiency alone in both the M-Wnt and metM-Wnt^{lung} cell lines resulted in moderate reduction in growth and colony formation over time, consistent with the weak effects of unaccompanied autophagy inhibition in the literature[56,57]. Even under nutrient replete conditions, it appears that autophagy deficiency causes accumulations of damaged mitochondria within the cancer cells. Decreased mitochondrial membrane polarization, reduced mitochondrial superoxide (a normal by-product of oxidative phosphorylation), and lowered maximum oxygen consumption rate all signal impairments in mitochondrial oxidative phosphorylation. Mitophagy, a selective method for mitochondrial degradation by autophagy, is not affected by ATG5 knockout, but appears to have a negligible effect on clearing damaged mitochondria in this case. On a cellular scale, damaged mitochondria impair energy generation and biosynthesis, but on a large tumor scale, accumulation of damaged mitochondria and autophagy deficiency are associated with benign oncocytomas rather than carcinomas in their autophagy competent counterparts[58]. Additionally, autophagy inhibition appeared to marginally induce EMT reversion, as seen by the downregulation of mesenchymal EMT transcription factors and a shift towards E-Cadherin over N-Cadherin, recognized markers of a mesenchymal to epithelial transition (MET)

[21,49,52]. While it is unclear whether EMT is dependent on autophagy or if EMT and autophagy act in parallel to promote invasive phenotypes, TGF-β signaling may drive autophagy inhibition's induction of an MET. Autophagy inhibition attenuates TGF-β signaling, and in one study hepatocellular carninomas required autophagy for TGF-β signaling[59,60]. TGF-β signaling induces EMT, and thus if TGF-β signaling in a steady state is decreased, EMT correspondingly reverses[59,60].

1% FBS media, in contrast to the 10% FBS media cells are usually cultured in, acts synergistically with autophagy inhibition to decrease growth. 1% FBS is additionally considered one of the best methods to mimic calorie restriction *in vitro,* as FBS contains amino acids, lipids, hormones, growth factors, and cytokines, it provides a more realistic systemic nutrient deprivation effect than restricting a singular nutrient[37,62]. The mechanism for 1% FBS's effects may not lie within the mTOR pathway that calorie restriction is believed to act through, as treatments with Metformin, an AMPK activator/mTOR inhibitor, and BMS-754807 had no combined effect with autophagy inhibition. However, AKT dependent, mTOR independent effects downstream of PI3K could be responsible for ATG5 knockout sensitization to 1% FBS. The PI3K/AKT pathway integrates multiple nutrient status, growth factor, and stress signals, all of which would be altered by 1% FBS

Of the chemotherapeutic agents assayed in combination with autophagy inhibition, Doxorubicin was the only agent to decrease survival synergistically with autophagy inhibition. Doxorubicin is also the only agent known to produce free radicals in addition to its primary cytotoxic mechanism as a DNA intercalating agent[63,64]. An inability of autophagy deficient cells to properly respond to increased ROS as a result of Doxorubicin induced free radicals may explain its unique effectiveness in these cells. Reactive oxygen species are a major regulator of

autophagy, and evidence shows that autophagy acts similar to a negative feedback loop where ROS-induced autophagy brings oxidative stress levels back down to a healthy level by removing damaged organelles[17]. In autophagy deficient cells, ROS cannot be brought back down as easily, and additional ROS formed as a result of Doxorubicin treatment induce apoptosis. This explanation tracks well with the general ROS flow cytometry data; despite basally having lower reactive oxygen species than the Wild-Type, the ATG5 knockouts have much higher ROS than the Wild-Type with Doxorubicin treatment, but not Gemcitabine treatment. Furthermore, the lower basal ROS in the ATG5 knockouts can be explained by NRF2 induction. Autophagy substrate p62 is upregulated with cellular stress and has the role of inducing autophagy under basal conditions. P62 also interacts with Keap1, a ubiquitin ligase involved in degrading NRF2, and competes for the NRF2 binding site, thus releasing NRF2 in p62 presence. Autophagy deficient cells cannot clear p62 and accumulate it, resulting in stabilized NRF2 and high transcriptional antioxidant activity[65,66]. With added exogenous stress however, p62 may be induced and accumulate at toxic levels, inducing additional oxidative and electrophilic stress in both NRF2 dependent and independent manners[65,66]. In short, autophagy deficient cells may have a decreased ability to respond to the additional oxidative stress of Doxorubicin treatment due to p62 accumulation without increased NRF2 antioxidant activity.

Conclusions

We conclude that autophagy inhibition combination therapy in Wnt driven, claudin-low Triple negative breast cancer does represent a strong potential avenue for adjuvant and neoadjuvant therapies with the addition of nutrient or chemotherapeutic stress. However, specific molecular targets that work in accord with autophagy must be identified if therapies are to be brought to practical levels of efficacy.

Works Cited

1. Siegel R, Miller K, Jemal A. Cancer Statistics. *CA Cancer J Clin*. 2017;67(1):7-30. doi:10.3322/caac.21387.

2. Howlader N, Noone AM, Krapcho M, Miller D, Bishop K, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ CK (eds). SEER Cancer Statistics Review, 1975-2013. *Natl Cancer Institute*. 2016.

3. Perou CM. Molecular stratification of Triple negative breast cancer. *Oncologist*. 2010;15(suppl 5):39-48. doi:10.1634/theoncologist.

4. American Cancer Society. Breast Cancer Facts & Figures 2017-2018. 2017:1-44. doi:10.1007/s10549-012-2018-4.Mesothelin.

5. Bianchini G, Balko JM, Mayer IA, Sanders ME, Gianni L. Triple-negative breast cancer: Challenges and opportunities of a heterogeneous disease. *Nat Rev Clin Oncol*. 2016;13(11):674-690. doi:10.1038/nrclinonc.2016.66.

6. Malorni L, Shetty PB, De Angelis C, et al. Clinical and biologic features of triple-negative breast cancers in a large cohort of patients with long-term follow-up,. *Breast Cancer Res Treat*. 2012;136(3):795-804. doi:10.1007/s10549-012-2315-y.

7. Curigliano G, Goldhirsch A. The triple-negative subtype: New ideas for the poorest prognosis breast cancer. *J Natl Cancer Inst - Monogr*. 2011;(43):108-110. doi:10.1093/jncimonographs/lgr038.

8. Iqbal J, Ginsburg O, Rochon PA, Sun P, Narod SA. Differences in Breast Cancer Stage at Diagnosis and Cancer-Specific Survival by Race and Ethnicity in the United States. *Jama*. 2015;313(2):165. doi:10.1001/jama.2014.17322.

9. Pal SK, Childs BH, Pegram M. Triple negative breast cancer: Unmet medical needs. *Breast Cancer Res Treat*. 2011;125(3):627-636. doi:10.1007/s10549-010-1293-1.

10. Pareja F, Geyer FC, Marchiò C, Burke KA, Weigelt B, Reis-Filho JS. Triple-negative breast cancer: the importance of molecular and histologic subtyping, and recognition of low-grade variants. *npj Breast Cancer*. 2016;2(1):16036. doi:10.1038/npjbcancer.2016.36.

11. Pohl S-G, Brook N, Agostino M, Arfuso F, Kumar AP, Dharmarajan A. Wnt signaling in triple-negative breast cancer. *Oncogenesis*. 2017;6(4):e310. doi:10.1038/oncsis.2017.14.

12. Ponti D, Costa A, Zaffaroni N, et al. Isolation and In vitro Propagation of Tumorigenic Breast Cancer Cells with Stem / Progenitor Cell Properties. *Cancer Res*. 2005;(13):5506-5512.

13. Dunlap SM, Chiao LJ, Nogueira L, et al. Dietary energy balance modulates epithelial-to-mesenchymal transition and tumor progression in murine claudin-low and basal-like mammary tumor models. *Cancer Prev Res*. 2012;5(7):930-942. doi:10.1158/1940-6207.CAPR-12-0034.

14. Zhan T, Rindtorff N, Boutros M. Wnt signaling in cancer. *Oncogene*. 2017;36(11):1461-1473. doi:10.1038/onc.2016.304.

15. Levine B, Kroemer G. Autophagy in the Pathogenesis of Disease. *Cell*. 2008;132(1):27-42. doi:10.1016/j.cell.2007.12.018.

16. Yoshii SR, Mizushima N. Monitoring and measuring autophagy. *Int J Mol Sci*. 2017;18(9):1-13. doi:10.3390/ijms18091865.

17. Poillet-Perez L, Despouy G, Delage-Mourroux R, Boyer-Guittaut M. Interplay between ROS and autophagy in cancer cells, from tumor initiation to cancer therapy. *Redox Biol*. 2015;4:184-192. doi:10.1016/j.redox.2014.12.003.

18. Kimmelman AC, White E. Autophagy and Tumor Metabolism. *Cell Metab*. 2017;25(5):1037-1043. doi:10.1016/j.cmet.2017.04.004.

19. Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. *Invit Rev J Pathol J Pathol*. 2010;221(February):3-12. doi:10.1002/path.2697.

20. Zhao J, Goldberg AL. Coordinate regulation of autophagy and the ubiquitin proteasome system by MTOR. *Autophagy*. 2016;12(10):1967-1970. doi:10.1080/15548627.2016.1205770.

21. Su Z, Yang Z, Xu Y, Chen Y, Yu Q. Apoptosis, autophagy, necroptosis, and cancer metastasis. *Mol Cancer*. 2015;14(1):1-14. doi:10.1186/s12943-015-0321-5.

22. Sui X, Chen R, Wang Z, et al. Autophagy and chemotherapy resistance: A promising therapeutic target for cancer treatment. *Cell Death Dis*. 2013;4(10):1-12. doi:10.1038/cddis.2013.350.

23. Yu-Long Hu, Michael DeLay, Arman Jahangiri, Annette M. Molinaro SD, Rose, W. Shawn Carbonell and MKA. Hypoxia-induced autophagy promotes tumor cell survival and adaptation to anti-angiogenic treatment in glioblastoma. 2009;27(4):339-351. doi:10.1016/j.neuron.2009.10.017.A.

24. White E. The role for autophagy in cancer. *J Clin Invest*. 2015;125(1):42-46. doi:10.1172/JCI73941.Autophagy.

25. Hewitt G, Korolchuk VI. Repair, Reuse, Recycle: The Expanding Role of Autophagy in Genome Maintenance. *Trends Cell Biol*. 2017;27(5):340-351. doi:10.1016/j.tcb.2016.11.011.

26. Lashinger LM, O'Flanagan CH, Dunlap SM, et al. Starving cancer from the outside and inside: Separate and combined effects of calorie restriction and autophagy inhibition on Ras-driven tumors. *Cancer Metab*. 2016;4(1):1-11. doi:10.1186/s40170-016-0158-4.

27. Kenific C, Thorburn A, Debnath J. Autophagy and Metastasis: Another double-edged sword. *Curr Opin Cell Biol*. 2010;64(5):645-662. doi:10.1016/j.neuron.2009.10.017.A.

28. Guo JY, Chen HY, Mathew R, et al. Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. *Genes Dev*. 2011;25(5):460-470. doi:10.1101/gad.2016311.

29. Mowers EE, Sharifi MN, Macleod KF. Autophagy in cancer metastasis. *Oncogene*. 2017;36(12):1619-1630. doi:10.1038/onc.2016.333.

30. Mehlen P, Puisieux A. Metastasis: A question of life or death. *Nat Rev Cancer*. 2006;6(6):449-458. doi:10.1038/nrc1886.

31. Weigelt B, Peterse JL, Van't Veer LJ. Breast cancer metastasis: Markers and models. *Nat Rev Cancer*. 2005;5(8):591-602. doi:10.1038/nrc1670.

32. Guo JY, Karsli-Uzunbas G, Mathew R, et al. Autophagy suppresses progression of K-ras-induced lung tumors to oncocytomas and maintains lipid homeostasis. *Genes Dev*. 2013;27(13):1447-1461. doi:10.1101/gad.219642.113.

33. Lambert AW, Pattabiraman DR, Weinberg RA. Emerging Biological Principles of Metastasis. *Cell*. 2017;168(4):670-691. doi:10.1016/j.cell.2016.11.037.

34. Kuma A, Hatano M, Matsui M, et al. The role of autophagy during the early neonatal starvation period. *Nature*. 2004;432(7020):1032-1036. doi:10.1038/nature03029.

35. Chude CI, Amaravadi RK. Targeting autophagy in cancer: Update on clinical trials and novel inhibitors. *Int J Mol Sci*. 2017;18(6). doi:10.3390/ijms18061279.

36. Yang YP, Hu LF, Zheng HF, et al. Application and interpretation of current autophagy inhibitors and activators. *Acta Pharmacol Sin*. 2013;34(5):625-635. doi:10.1038/aps.2013.5.

37. O'Flanagan CH, Smith LA, McDonell SB, Hursting SD. When less may be more: Calorie restriction and response to cancer therapy. *BMC Med*. 2017;15(1):1-9. doi:10.1186/s12916-017-0873-x.

38. Saleh AD, Simone BA, Palazzo J, et al. Caloric restriction augments radiation efficacy in breast cancer. *Cell Cycle*. 2013;12(12):1955-1963. doi:10.4161/cc.25016.

39. Mattison JA, Colman RJ, Beasley TM, et al. Caloric restriction improves health and survival of rhesus monkeys. *Nat Commun*. 2017;8(May 2016):1-12. doi:10.1038/ncomms14063.

40. Schroll MM, LaBonia GJ, Ludwig KR, Hummon AB. Glucose Restriction Combined with Autophagy Inhibition and Chemotherapy in HCT 116 Spheroids Decreases Cell Clonogenicity and Viability Regulated by Tumor Suppressor Genes. *J Proteome Res*. 2017;16(8):3009-3018. doi:10.1021/acs.jproteome.7b00293.

41. Garufi A, Ricci A, Trisciuoglio D, et al. Glucose restriction induces cell death in parental but not in homeodomain-interacting protein kinase 2-depleted RKO colon cancer cells: Molecular mechanisms and implications for tumor therapy. *Cell Death Dis*. 2013;4(5):1-11. doi:10.1038/cddis.2013.163.

42. Thorburn A, Thamm DH, Gustafson DL. Autophagy and Cancer Therapy. *Mol Pharmacol Mol Pharmacol*. 2014;85(June):830-838. doi:10.1124/mol.114.091850.

43. Debnath J, Baehrecke EH, Kroemer G. Does autophagy contribute to cell death? *Autophagy*. 2005;1(2):66-74. doi:10.4161/auto.1.2.1738.

44. Yoshimori T. Autophagy: Paying Charon's Toll. *Cell*. 2007;128(5):833-836. doi:10.1016/j.cell.2007.02.023.

45. O'Flanagan CH, Rossi EL, McDonell SB, et al. Metabolic reprogramming underlies metastatic potential in an obesity-responsive murine model of metastatic triple negative breast cancer. *npj Breast Cancer*. 2017;3(1):26. doi:10.1038/s41523-017-0027-5.

46. Ryan J, Letai A. BH3 profiling in whole cells by fluorimeter or FACS. *Methods*. 2013;61(2):156-164. doi:10.1016/j.ymeth.2013.04.006.

47. Marchetti C, Obert G, Deffosez A, Formstecher P, Marchetti P. Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. *Hum Reprod*. 2002;17(5):1257-1265. doi:10.1093/humrep/17.5.1257.

48. Barth S, Glick D, Macleod KF. Autophagy: Assays and artifacts. *J Pathol*. 2010;221(2):117-124. doi:10.1002/path.2694.

49. Nieto MA, Huang RYYJ, Jackson RAA, Thiery JPP. Emt: 2016. *Cell*. 2016;166(1):21-45. doi:10.1016/j.cell.2016.06.028.

50. Wu Y, Zhou BP. Snail: More than EMT. *Cell Adhes Migr*. 2010;(June):199-203.

51. Khan A, Chen H. Twist : a molecular target in cancer therapeutics. *Tumor Biol*. 2013:2497-2506. doi:10.1007/s13277-013-1002-x.

52. Brabletz T, Kalluri R, Nieto MA, Weinberg RA. EMT in cancer. *Nat Rev Cancer*. 2018. doi:10.1038/nrc.2017.118.

53. Larsen JE, Nathan V, Osborne JK, et al. ZEB1 drives epithelial-to-mesenchymal transition in lung cancer. *J Clin Invest*. 2016;126(9):3219-3235. doi:10.1172/JCI76725.

54. Satelli A, Li S. Vimentin in cancer and its potential as a molecular target for cancer therapy. *Cell Mol Life Sci*. 2011;68(18):3033-3046. doi:10.1007/s00018-011-0735-1.

55. Ramis-conde I, Chaplain MAJ. Multi-scale modelling of cancer cell intravasation : the role of cadherins in metastasis. *IOP Phys Biol*. 2009. doi:10.1088/1478-3975/6/1/016008.

56. Xu XD, Zhao Y, Zhang M, et al. Inhibition of autophagy by deguelin sensitizes pancreatic cancer cells to doxorubicin. *Int J Mol Sci*. 2017;18(2). doi:10.3390/ijms18020370.

57. Aydinlik S, Erkisa M, Cevatemre B, et al. Enhanced cytotoxic activity of doxorubicin through the inhibition of autophagy in triple negative breast cancer cell line. *Biochim Biophys Acta - Gen Subj*. 2017;1861(2):49-57. doi:10.1016/j.bbagen.2016.11.013.

58. Zong W, Rabinowitz JD, White E. Review Mitochondria and Cancer. *Mol Cell*. 2016;61(5):667-676. doi:10.1016/j.molcel.2016.02.011.

59. Xu J, Lamouille S, Derynck R. TGF-B-induced epithelial to mesenchymal transition. *Cell Res*. 2009;19(2):156-172. doi:10.1038/cr.2009.5.

60. Kiyono K, Suzuki HI, Matsuyama H, et al. Autophagy is activated by TGF-β and potentiates TGF-β-mediated growth inhibition in human hepatocellular carcinoma cells. *Cancer Res*. 2009;69(23):8844-8852. doi:10.1158/0008-5472.CAN-08-4401.

61. Longo VD, Fontana L. Calorie restriction and cancer prevention: metabolic and molecular

mechanisms. *Trends Pharmacol Sci*. 2010;31(2):89-98. doi:10.1016/j.tips.2009.11.004.

62. Blagosklonny M V. Calorie restriction: Decelerating mTOR-driven aging from cells to organisms (including humans). *Cell Cycle*. 2010;9(4):683-688. doi:10.4161/cc.9.4.10766.

63. Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L. Anthracyclines : Molecular Advances and Pharmacologic Developments in Antitumor Activity and Cardiotoxicity. *Pharmacol Rev*. 2004;56(2):185-229. doi:10.1124/pr.56.2.6.185.

64. Mccord JM. Free Radicals : The Pros and Cons of Antioxidants Iron , Free Radicals , and Oxidative Injury. *Blood*. 2004;(April):3171-3172.

65. Rusten TE, Stenmark H. P62, an autophagy hero or culprit? *Nat Cell Biol*. 2010;12(3):207-209. doi:10.1038/ncb0310-207.

66. Komatsu M, Kurokawa H, Waguri S, et al. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nat Cell Biol*. 2010;12(3):213-223. doi:10.1038/ncb2021.